THE STAPHYLOCOCCAL PEP DEPENDENT PHOSPHOTRANSFERASE SYSTEM, PROTON MAGNETIC RESONANCE (PMR) STUDIES ON THE PHOSPHORYL CARRIER PROTEIN HPr: EVIDENCE FOR A PHOSPHOHISTIDINE RESIDUE IN THE INTACT PHOSPHO-HPr MOLECULE

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1. Introduction

In many micro-organisms several carbohydrates are transported into the cell by vectorial phosphorylation via the PEP dependent phosphotransferase system (PTS) [1,2]. In staphylococcus aureus the PTS has been studied in detail. The following reaction sequence occurs during phosphorylation of galactosides [3]:

$$\text{PEP} + \text{Enzyme I} \xrightleftharpoons{Mg^{2+}} \text{P-Enzyme I} + \text{Pyruvate}$$

$$\text{P-Enzyme I} + \text{HPr} \rightleftharpoons \text{HPr} + \text{Enzyme I}$$

$$\text{P-HPr} + 1/3\ \text{Factor III} \rightleftharpoons 1/3\ \text{P-Factor III} + \text{HPr}$$

$$1/3\ \text{P-Factor III} + \text{Galactoside} \xrightarrow[\text{Enzyme II}]{Mg^{2+}} \text{Galactoside-6-P} + 1/3\ \text{Factor III}$$

Enzyme I 80 000 daltons
HPr 8500 daltons
} constitutive soluble proteins

Factor III (FIII) 3 × 11 000 daltons soluble
Enzyme II membrane bound
} Galactoside specific inducible proteins

The phosphorylated forms of the P-carrier proteins HPr and F III can be demonstrated using ^{32}P PEP as phosphoryl donor. After alkaline hydrolysis in 3 M NaOH 100°C, 3 hr 1-[^{32}P]histidine was isolated from ^{32}P-HPr whereas 3-[^{32}P]histidine was isolated from ^{32}P-F III, leading to the conclusion that the phosphoryl group in HPr was linked to the N-1 of the imidazol ring of the histidine residue in the protein [4,5]. However it could not be excluded that phosphoryl migration had occurred from an unidentified residue in HPr to the histidine residue, where the phosphoryl group is stable towards the extreme conditions of alkaline hydrolysis. Therefore we used PMR spectroscopy, a method well suited to detect modifications on the imidazol ring of histidine residues in intact proteins [6] in order to demonstrate phosphorylation of the histidine residue in intact HPr.

2. Materials and methods

2.1. *Preparation of PTS components*

HPr: HPr was isolated from crude extracts of the strain S 305 A as described [4]. The protein was homogeneous on acrylamide gels at pH 9.3 and in the presence of SDS.

Enzyme I: A crude preparation of Enzyme I was used as described previously [3]. The specific activity was about 5 units per mg protein (1 unit = 1 μM orthonitrophenol released in 10 min at 37°C in the extract complementation assay as described earlier) [7].

2.2. *Phosphorylation of HPr*

18 units of Enzyme I in 350 μl (0.05 M Tris–HCl, pH 7.5, 10^{-4} M EDTA 0.1% mercaptoethanol) 5 μl 1 M $MgCl_2$, 50 μl 0.5 M PEP pH 7.5 and 20 mg of

freeze dried purified HPr was incubated for 10 min at 37°C, then again 50 μl PEP were added followed by 10 min incubation. The density of the reaction mixture was increased by the addition of 200 μl glycerol. Then the solution was chromatographed on a Sephadex G75 column (1 X 40 cm) equilibrated with 0.1 M $Na_2B_4O_7$ pH 9.3 (suprapure Merck) in D_2O (99.75%). 5 μl aliquots of the collected fractions (0.7 ml) were tested with the complementation assay described [7]. Fractions containing HPr activity were pooled and concentrated to about 0.6 ml with an Amicon pressure dialysis apparatus (Amicon model 12). The sample was kept frozen until it was used for PMR spectroscopy to prevent hydrolysis of P-HPr.

2.3. *Determination of phosphorylated and non-phosphorylated HPr*

P-HPr can be separated from HPr by acrylamide gel electrophoresis at pH 9.3 0.4 M Tris–glycin [8]. The R_f value (reference dye: Bromophenol Blue) for HPr was 0.66 for P-HPr 0.77 at an acrylamide concentration of 7.5%. The intensity of the stained bands (Coomassie Blue) was estimated with an Isco gel scanning device.

2.4. *PMR spectra*

The proton magnetic resonance (PMR) spectra were observed with Bruker HX-spectrometer at 90 MHz modified for Fourier transform operation. The 270 MHz spectra were obtained at a Bruker spectrometer which has been kindly made available to us by Professor Ruterjans, University of Münster, Germany.

3. Results and discussion

3.1. *PMR spectrum of HPr*

Spectra were taken in D_2O in order to remove signals from exchangeable protons. The amino acid composition of HPr (see table 1) predicts signals in the aromatic region from just the following residues. 1 histidine, 1 phenylalanine and 3 tyrosines. Only the peak due to the C-2 proton of the imidazol ring is well resolved at 90 MHz and can be identified clearly by its pH-titration (fig.2) with a pk-value of 6.2. The PMR-spectrum of HPr at pH 9.2 is shown in fig.1a with improved resolution at 270 MHz.

Table 1
Aminoacid composition of staphylococcal HPr

Amino acid	Number of residues per molecule
Asp	7.49
Thr	4.77
Ser	4.97
Glu	8.53
Pro	0.99
Gly	6.0
Ala	6.38
Val	5.30
Met	3.50
Ile	6.50
Leu	5.34
Tyr	3.06
Phe	1.16
Lys	6.0
His	0.92
Arg	0.95
Cys	0
Trp	0

3.2. *PMR spectrum of phospho-HPr*

The spectrum of the phosphorylated sample is presented in fig.1b. The most obvious change concerns the C-2 proton peak on the low field side which

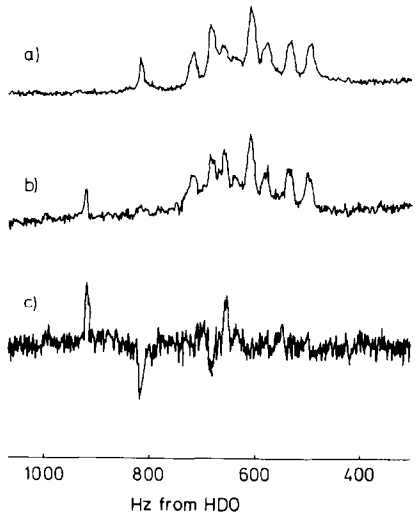

Fig.1. 270 MHz-PMR spectra in D_2O, 0.1 M $Na_2B_4O_7$ buffer, pH 9.3, 1000 scans. Shifts are given in Hz with respect to HDO. a) 35 mg/ml HPr. b) after enzymatic phosphorylation. c) difference spectrum b)–a).

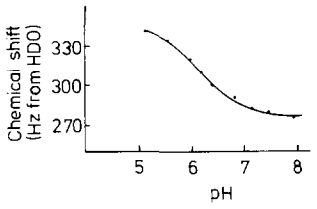

Fig.2. pH-titration of the C-2 proton peak in the 90 MHz-PMR spectrum of 70 mg/ml HPr in D_2O. Shifts are given in Hz with respect to HDO.

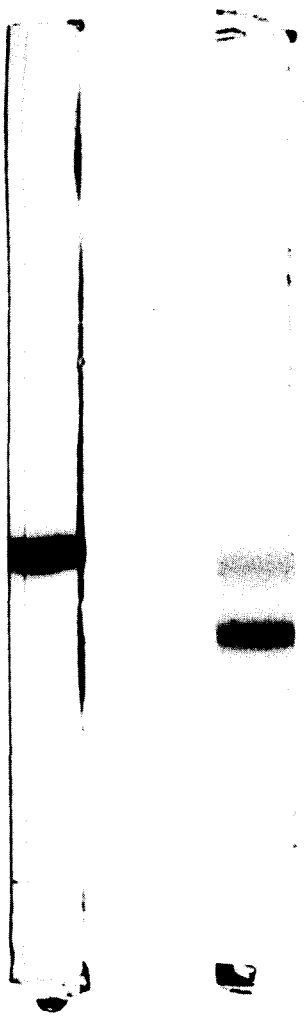

Fig.3. Acrylamide gel electrophoresis of HPr and phospho-HPr at pH 9.3. Left gel: pure HPr, right gel: Electrophoresis of P-HPr after Sephadex G75 chromatography.

is shifted downfield in the phosphorylated HPr. The difference spectrum shown in fig.1c indicates only one additional change in the aromatic region. A small upfield shift is observed at the position identified as the C-4 histidine proton by its pH-titration. From the spectra in fig.1 one concludes a nearly complete phosphorylation. The amount of P-HPr in the sample can be measured independently by the electrophoretic procedure described above and has been determined to be 85% (see fig.3) in good agreement with the magnetic resonance result.

3.3. *Evidence for 1-phosphohistidine residue in the P-HPr molecule*

The shifts of the C-2 and C-4 proton peak due to phosphorylation have been studied earlier for the model compound 3-phosphohistidine [9]. Only small shifts ($\leqslant 0.1$ ppm) have been observed. Since no PMR data of 1-P-histidine were available, we investigated the phosphorylation of histidine by phosphoamidate in more detail: The reaction has been followed by both PMR and paper-electrophoresis [10]. Our data for the 3-P-histidine confirms the above results. The 1-P-histidine is characterized by a downfield shift of about 0.3 ppm for the C-2 proton peak as compared to the free histidine spectrum. The agreement with the shift of about 100 Hz between the C-2 peaks of histidine residue in P-HPr and HPr in fig.1 is excellent.

The experiments described above present strong evidence, that the phosphoryl-group in the native P-HPr molecule is linked to the N-1 of the imidazol ring of the only histidine residue.

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References

[1] Kaback, H. R. (1970) Ann. Rev. Biochem. 39, 561–599.

[2] Roseman, S. (1969) J. Gen. Physiol. 54, 138–183s.

[3] Stein, R., Schrecker, O. and Hengstenberg, W. (1974) FEBS Lett. 42, 98–100.
[4] Schrecker, O., Merkle, G. and Hengstenberg, W. (1974) submitted to Eur. J. Biochem.
[5] Simoni, R. D., Hays, J. B., Nakazawa, T. and Roseman, S. (1973) J. Biol. Chem. 248, 957–965.
[6] Roberts, G. C. K. and Jardetzky, O. (1970) Adv. Protein Chem. 24, 447–545.
[7] Hengstenberg, W., Penberthy, W. K., Hill, K. L. and Morse, M. L. (1969) J. Bacteriol. 99, 383–388.
[8] Hjerten, S., Jerstedt, S. and Tiselius, A. (1965) Anal. Biochem. 11, 219–223.
[9] Hultquist, D. E., Moyer, R. W. and Boyer, P. D. (1966) Biochemistry 5, 322–331.
[10] Schrecker, O. and Gassner, M. (unpublished results).